A technique to study the development in wood of soft rot fungi and its application to *Ceratocystis ulmi*

F. CASAGRANDE
CIBA AG, Basel 4000, Switzerland

AND

G. B. OUELLETTE
Forest Research Laboratory, Department of Fisheries and Forestry,[1] *Quebec, Quebec*

Received April 13, 1970

CASAGRANDE, F., and G. B. OUELLETTE. 1971. A technique to study the development in wood of soft rot fungi and its application to *Ceratocystis ulmi*. Can. J. Bot. **49**: 155–159.

The development of fungi causing soft rots and of ensuing cavities in cell walls was studied with a new technique, consisting in inoculation of sterilized wood sections placed on agar slants. The senior author proposes a new system of classification of the soft rot fungi. Applied to investigations on *Ceratocystis ulmi* (Buism.) C. Moreau, this technique permits further characterization of the morphology of this fungus, including the formation of microhyphae and microspore-like particles, often less than 0.5 μ in diameter, and recognition of the various configurations brought about by its growth in wood tissues. For example, microhyphae grow in a reticulate manner in close contact with starch grains, and these seem to break down.

A l'aide d'une nouvelle méthode, nous avons suivi l'évolution de plusieurs champignons agents de pourriture molle du bois et des cavernes qu'ils forment. Cette méthode consiste à inoculer des coupes de bois stérilisées et placées sur un milieu gélosé en tube, dans les conditions normales d'aseptie. A la lumière des résultats obtenus, le premier auteur propose une nouvelle classification des champignons responsables de cette destruction du bois. Appliquée à l'étude du *Ceratocystis ulmi* (Buism.) C. Moreau, cette méthode a permis de montrer nettement la formation de microspores et de microhyphes souvent de moins de 0.5 μ de diamètre, le réseau ténu des microhyphes entourant les grains d'amidons, qui apparemment se désintègrent, et la grande diversité de formes qu'engendre la croissance du champignon dans les tissus.

Introduction

Soft rot of wood is a term which applies to deterioration brought about by Ascomycetes and imperfect fungi, as opposed to the brown and white rots, which are usually caused by basidiomycetes. This deterioration has been known for more than a century (3), but it was in 1937 (1) that fungi were implicated as causal agents. In the past 15 years, different aspects of the problem were studied in detail (2, 3, 4, 5, 6, 13, 16). Liese (4, 5), with the help of both the light and electron microscopes, studied the mode of action of these fungi and the development of cavities in the cell wall. He reported the presence of microhyphae and "borhyphae" of less than 0.5 μ in diameter in soft rot and blue-stain fungi (7). Microhyphae were also reported in other fungi (9, 12, 15). Liese observed a zone of degradation of enzymatic origin, around these small hyphae. Cavities eventually resulted in the secondary and (or) tertiary wall, depending on the host. Courtois (2) proposed a classification of the soft rot fungi in 14 groups on the basis of cavity origin and morphology. However, it was not clearly established whether these types of cavities could actually represent different stages of cavity development.

In an effort to understand better the development of these fungi in wood and the conditions such as humidity, aeration, and nutrition (13, 16) favoring this development, a new technique was developed by the senior author. Description of this technique along with results of its application by the junior author to a morphological study of *Ceratocystis ulmi* (Buism.) C. Moreau in elm wood are the object of the present paper.

Method

Wood sections are cut in the plane desired, from blocks about 1 cm square or from branch sections, using a microtome set at 15 μ. Sections are sterilized by dry heat at 100°C for 5 h or in propylene oxide for 12 h (for studies with *C. ulmi*). The nutritive medium is made up as follows: peptone, 1 g; KH_2PO_4, 1 g; $MgSO_4$, 500 mg; malt extract, 2 g; agar, 15 g; and H_2O, 1000 ml (organic compounds, Difco). Slants containing this medium are prepared and sterilized in an autoclave in the usual manner. Under aseptic conditions, a wood section is transferred onto the solidified medium, and inoculated with the desired fungus.

[1]P.O. Box 3800, Quebec 10.

Incubation takes place under the conditions desired. Examinations are made either with living tissues unstained or treated with vital stains, or with fixed preparations stained with cotton blue in lactophenol. For permanent mounts, the sections are left in the staining solution for 2 days, rinsed in distilled water, dehydrated in a series of 10, 20, 40, 60, 80, and 100% Dioxan, and then mounted in Canada balsam or other mounting medium such as Caedax. Various phases in the formation of cavities were studied as follows: sterilized sections were mounted on agar cubes on slides and inoculated; marked areas were then examined periodically under phase contrast or after treatment with vital stains. In studies with *C. ulmi*, the culture used was an isolate obtained July 1968 from a diseased American elm at St-Romuald near Quebec city. For purpose of comparison, a culture of *Ceratocystis minor* (Hedgc.) Hunt (isolated September 1968, from *Pinus montana Miller*, Swiss National Park) was also used in these tests. Samples of European elm (*Ulmus campestris* L.) collected near Zürich, November 1968, and kept for 1 month at −2°C, and freshly collected samples of beech and Norway spruce (*Picea abies* Karst.) were used as test material.

Observations

(A) Fungi Causing Soft Rots

These fungi produced cavities of various lengths in secondary walls. The cavities were pointed at both ends, and circular to elliptical in cross section (Figs. 1–3) and originated from microhyphae 0.2–0.6 μ in diameter. These microhyphae, which ordinarily arose from hyphae of various sizes in adjacent cells (Figs. 4, 5, 41), abruptly bifurcated and branched within the cell wall or grew across the contiguous wall layers into neighboring cells. Although the microhyphae can grow freely in the wall (Fig. 1, arrow), the cavities seem to be oriented in the direction of micellae (Figs. 2, 3). Cavities obviously result from the degradation of the cell wall around microhyphae which eventually may become larger (Fig. 6). This area of degradation is indicated by a zone which may become light blue around the hyphae.

As shown by observations in the living state, microhyphae grow into the wall for a short distance before initiating cavities (Fig. 7). During the formation of cavities the microhyphae do not elongate, but they resume growth at one or both extremities of the cavity, which results in the formation of secondary and tertiary cavities. These are commonly separated from one another but they may coalesce (Fig. 2).

With this technique and wood of European beech (*Fagus sylvatica* L.) and Norway spruce it was possible to group, as shown below, 27 fungi suspected of causing soft rots. These fungi were either isolated in pure culture by the senior author from various samples of deteriorated wood or obtained from the stock culture collection of Dr. E. Müller, ETH (indicated thus by an *).

(a) Forming Cavities in Cell Walls of Both Beech and Spruce Wood

Represented (1) in Ascomycetes, in *Chaetomium*, by

**C. funicola* Cke., **C. globosum* Kunze, **C. gracile* Udagawa, **C. spinosum* Zopf., **C. spirale* Zopf., and **C. murorum* Corda; and

(2) in Hyphomycetes, by *Bisporomyces chlamydosporis* van Beyma, *Doratomyces nanus* (Ehrb. & Link) Morton & Smith and *D. stemonitis* (Pers. ex Fr.) Morton & Smith, *Graphium penicillioides* Corda, *Humicola grisaea* Traaen, *Margarinomyces microsperma* (Corda) Mangenot, **Trichurus spiralis* Hasselbring, *Gliomastix murorum* (Corda) Hughes, and *Gonatobotrys simplex* Corda.

(b) Forming Cavities in Cell Walls of Beech Only, but Causing Perforations (by Microhyphae) in Cell Walls of Spruce Wood

Represented in Ascomycetes, by

**Chaetomium homopilatum* Omvik., *Myxotrichum cancellatum* Philipps, and in Hyphomycetes, by *Phialophora lignicola* (Nannf.) Goidanich, **Phialophora richardsiae* (Nannf.) Conant, and **Scopulariopsis brevicaulis* (Sacc.) Bainier.

(c) Causing Perforations Only in Cell Walls of Both Beech and Spruce Wood

Represented in Ascomycetes, by

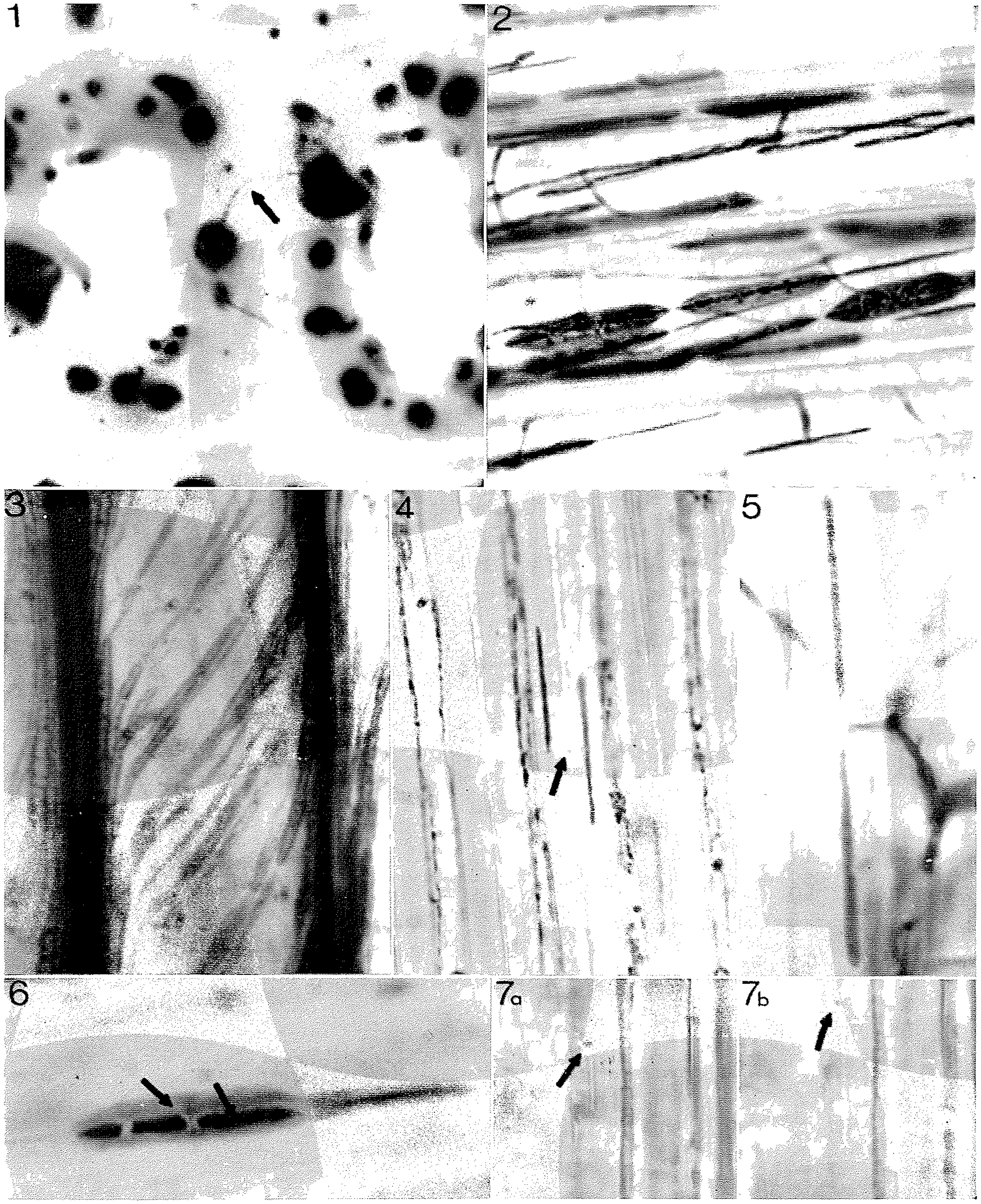
**Xylaria hypoxylon*, L. ex Grev., **Herpotrichia juniperi* (Duby) Petr., **Endothia parasitica* (Murr.) And., and **Nectria* sp. (from Poona, India), and in Hyphomycetes, by *Alternaria tenuissima* (Fr.) Wiltsh., **Pestalotia palmarum* Cooke, and *Verticillium albo-atrum* Rke. et Berth.

This classification is not in agreement with that of Courtois (2), who apparently based his classification on different stages of cavity development.

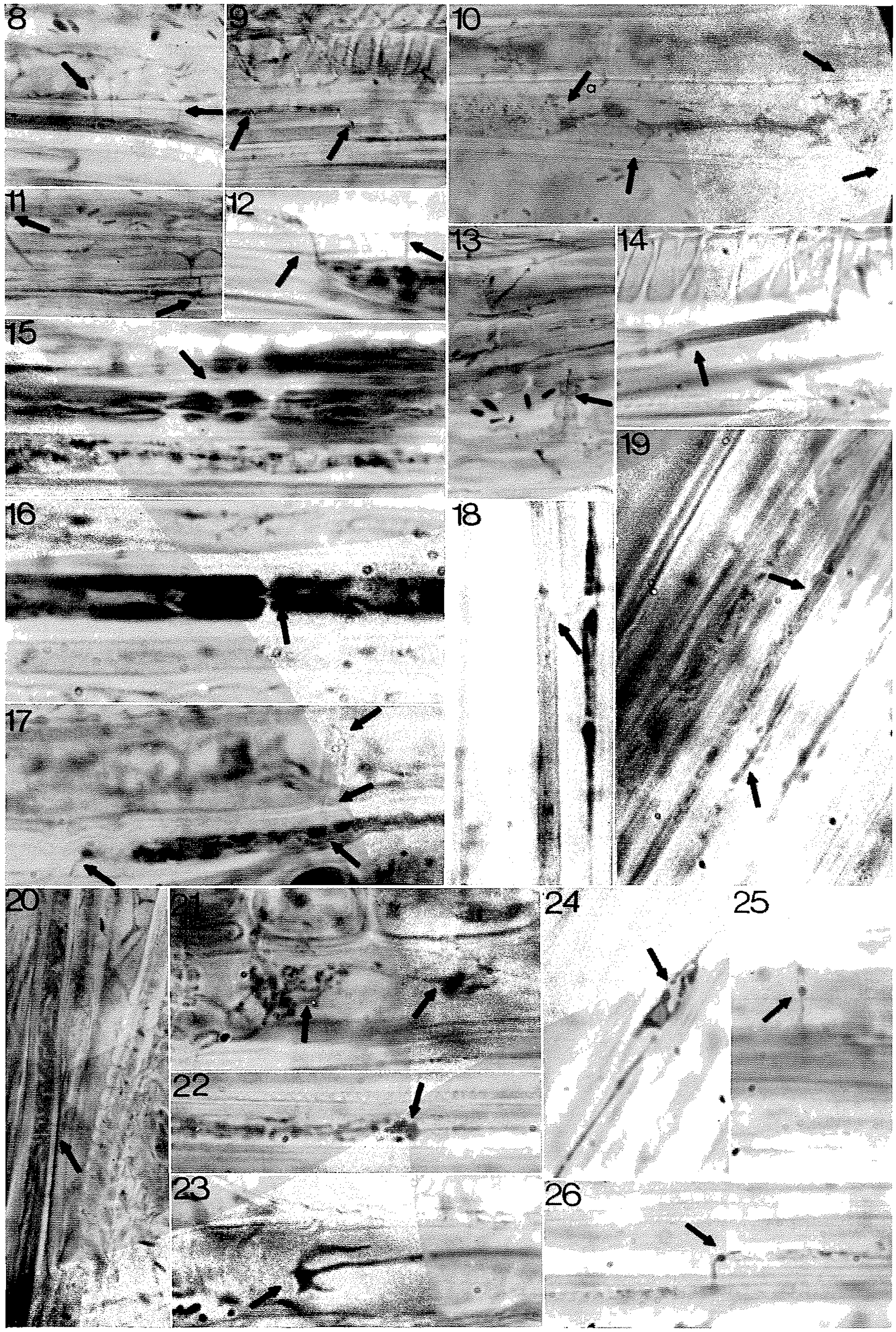
(B) Morphology of Ceratocystis ulmi

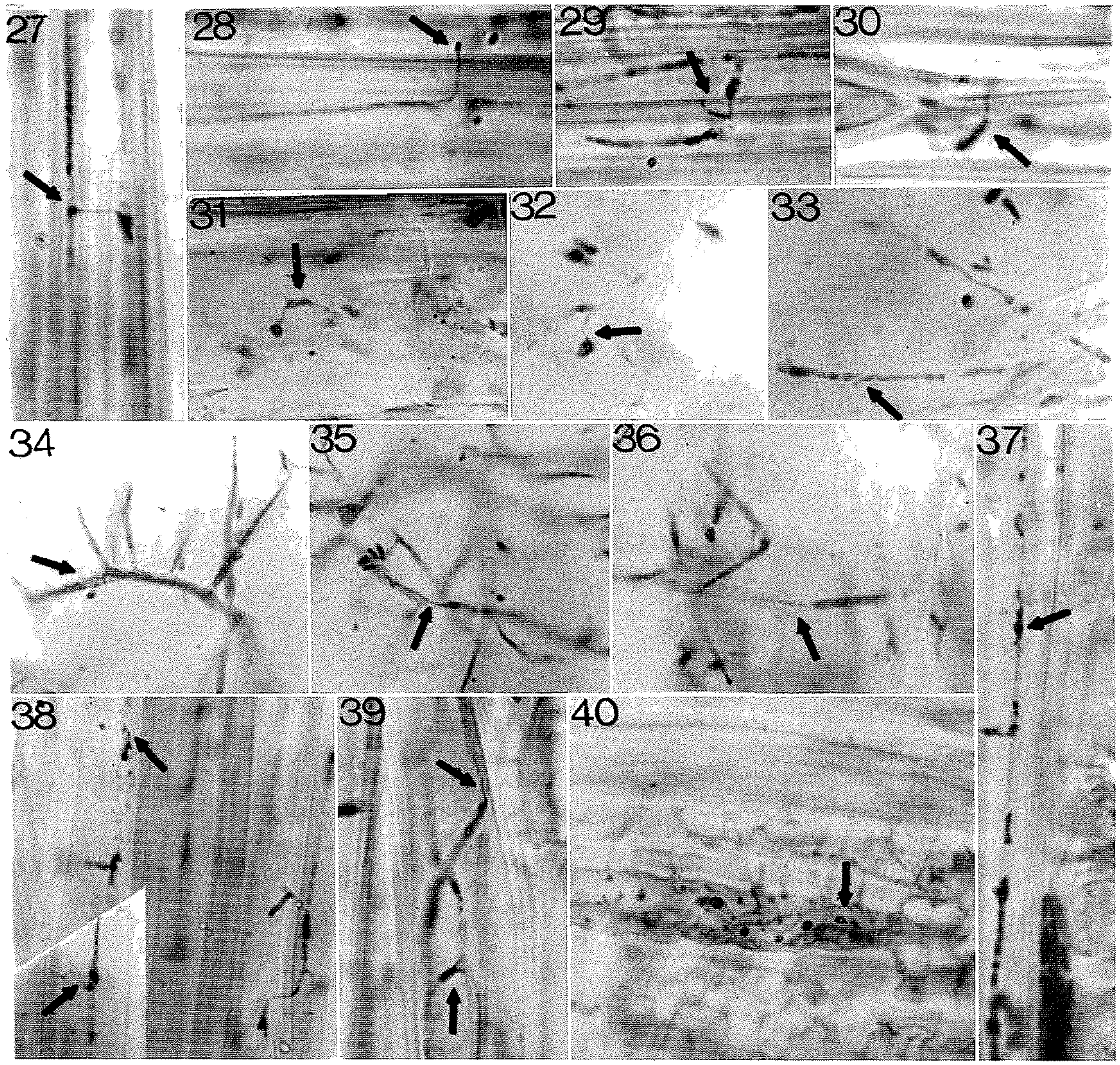
I. Observations

In sections of elm wood, *C. ulmi* invaded all tissues by means of numerous microhyphae 0.2–0.5 μ in diameter and often of considerable length. These microhyphae commonly crossed



FIGS. 1–7. Sections from spruce wood, stained with cotton blue, dehydrated, and mounted in Caedax, 7 days after inoculation. Fungi representative of group A. Fig. 1. Microhyphae growing in an apparently free direction from cavities in the secondary walls (arrow). Cross section, approx. 1000 ×. Figs. 2–3. Typical cavities at different stages of development. Approx. 600 ×. Figs. 4–5. Bifurcating microhyphae in secondary wall. In 4, initiating cavities. Approx. 600 × and 1200 × respectively. Fig. 6. Swollen hypha in a cavity with a zone of reaction around it (arrows). Approx. 1200 ×. Fig. 7. Partial sequence in hyphal growth and cavity formation in wall, phase contrast. Approx. 600 ×. In *a*, hyphal penetration; in *b*, 24 h later.


8
9
10
a
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26



FIGS. 8–40. Development of *Ceratocystis ulmi* in elm wood. Figs. 12, 15–16, 27, and 30, approx. 900 ×, others 1100 ×. Figs. 10, 12, 15, 17–30, and 37–39, from preparations made 7 days after inoculation, dehydrated, cotton blue; Figs. 8, 9, 11, 14, 16, 31–35, and 40, 7 days after inoculation, not dehydrated, cotton blue. Fig. 8. Microhyphae crossing through cell walls and forming microspore-like structures in other cell (arrow). Figs. 9–13. Microhyphae 0.5 μ in diameter or sometimes less in cells or growing through and between cell walls (arrows). In 9, microhyphae almost nonstaining, crossing wall; in 10, also microspore-like bodies (*a*); in 12, microhyphae (continuous with large mycelium) growing through walls of a fiber, one microhypha linked with spherical body (arrow, right); and in 13, microhyphae growing through end walls of two parenchyma cells next to a small vessel. Fig. 14. Zone of reaction around a microhypha (arrow). Figs. 15–18. Stages in cavern-like developments within fibers. In 15–16, reaction zones around hyphae which are swollen at points and contain endospore-like bodies (16, arrow); in 17–18, microhyphae of less than 0.5 μ (arrow) coming from hyphae (in 17 with wavy appearance) present in cavity-like formation. Figs. 19–20. Frequent branching and or anastamosing of hyphae in fibers (arrows). In 19, still surrounded at places by blue staining material of reaction zone (arrow, bottom). Fig. 21. Frequent branching and anastamosing (arrows) of microhyphae in a ray cell. Figs. 22–24. Types of configurations (arrows) resulting from swelling, branching, and anastamosing of hyphae within fibers. Figs. 25–27. Microhyphae (arrows) originating from spherical bodies within hyphae. Figs. 28–30. Microhyphae crossing cell walls and giving rise to spore-like bodies (arrows). Figs. 31–32. Larger free spores giving rise to microhyphae (arrows). Figs. 33–35. Microendohyphae formed from condensed protoplasm. In places, these have grown through enclosing hyphae (arrows). In 33, note microspore-like bodies also present in hyphae; in 35, two parallel microhyphae, one with a septation (arrow); and in 36, one microhypha formed by a kind of proliferation (arrow). Figs. 37–39. Condensed portions of protoplasm within hyphae (arrows); note apparent rigidity of these structures. In 38 and 39 particles linked with tiny filaments. Fig. 40. Portions of protoplasm, apparently free (arrow), associated with small filaments, or showing a type of budding.

cell walls or grew between them (Figs. 8–13). The fungus occasionally grew within the walls themselves, but typical cavities were not commonly observed in this instance. However, reaction zones similar to cavities occurred around small hyphae within fibers, and along or between cell walls (Figs. 14–16). In apparently further stages of development the tiny filaments or portions thereof enlarged and produced other numerous small hyphae and bodies resembling spores (Figs. 17–20). In addition to these, the "pseudocavities" contained amorphous or granular material considered to be either a secretion of the fungus or a degradation product of its mycelium. In longitudinal parenchyma and ray cells the fungus produced numerous microhyphae which branched in all directions, anastomosed frequently, and commonly followed the contour of starch grains. These cells thus showed an intricate reticulation and starch grains could no longer be distinguished, probably because they had disintegrated. In addition to repeated branching (Figs. 20–21), hyphae had a pronounced tendency to grow in a zigzag or helicoidal manner and to swell in whole or in part giving rise to web-like, dendrophysoid, neuroid, or other configurations (Figs. 21–24). Hyphae with very large cells and brown-pigmented walls acted as the point of origin of coremia which sometimes formed on the sections.

Microhyphae were often clearly observed to be associated with small spherical bodies. These spherical bodies represented often a point of origin, as indicated when they were within hyphae (Figs. 12, 25–27), but in many instances the direction of growth could not be established, while in others, the spherical bodies were homologous to a spore in formation (Figs. 28–30).

Larger spores also gave rise to microhyphae but not necessarily as a result of a physical stimulus, since small hyphae also formed from spores and hyphae free of contact with cell walls (Figs. 31–32). Microendohyphae of minute diameter formed within hyphae, from portions of apparently condensed protoplasm (Figs. 32–35), or through some kind of proliferation (Fig. 36). Microendohyphae were often observed to have grown through the wall of the parent hyphae. Condensation and fragmentation of the protoplasm were a general feature in certain types of hyphae, yielding structures which seemed capable of growth (Figs. 37–39). Because of their small size, it could not be established if such structures had formed a new wall, but this possibility was indicated by their apparent rigidity and their diameter being commonly larger than that of the enclosing hyphae. This,

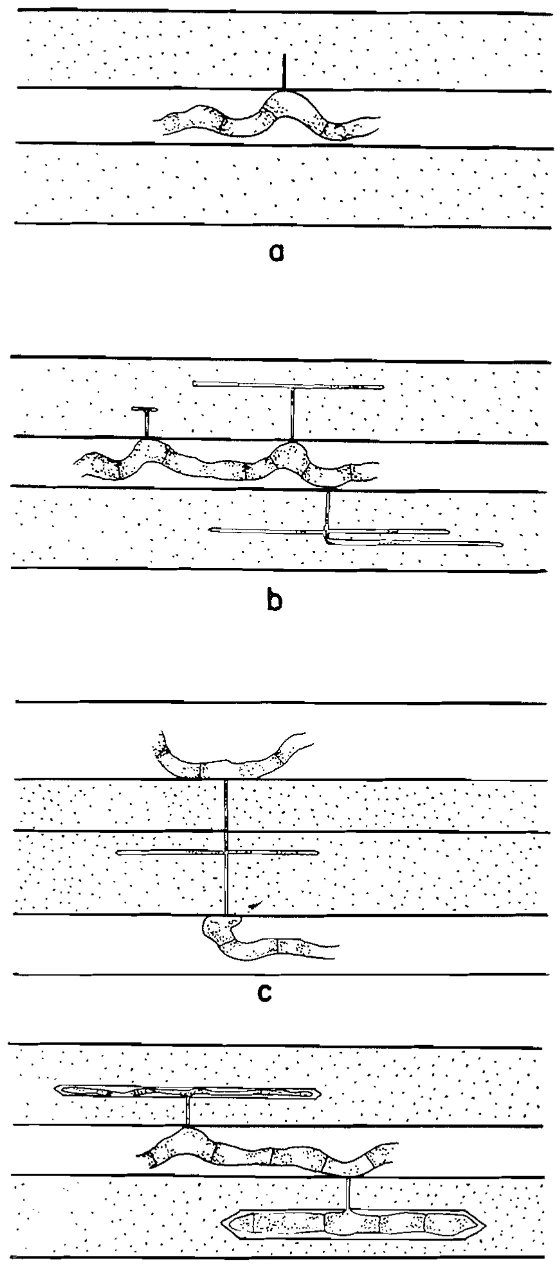


FIG. 41. Drawings representing different types of microhyphal growth and cavity formation (soft rot fungi).

together with the fact that other cytoplasmic particles were present between or around these structures eliminates plasmolysis, at least of sudden occurrence, as one of the factors responsible for their formation. Free structures of similar appearance (Fig. 40) were considered to be of endogenous origin.

In beech wood, *C. ulmi* also formed numerous microhyphae, typical cavities, and a large zone of reaction around some hyphae. Hyphae also had a tendency to branch frequently, to swell, and to form long chains of endospores, often by apparent fragmentation of the protoplasm. In spruce wood, *C. ulmi* rarely produced small hyphae and growth through cell walls was rare. The fungus also had a tendency to form endospores. On the other hand, in elm tissues, *C. minor* formed microhyphae, imparting to the ray cells a reticulate appearance. Starch grains, however, remained intact. Finally, it should be mentioned that starch grains were not observed in beech wood, probably because they had already disappeared at time of sampling.

II. Discussion

The present observations clarify further those expressed in previous reports by the junior author on the morphology and development of *C. ulmi* in elm trees (8, 9, 10) particularly (1) the formation of microhyphae and their growth in all xylem tissues and (2) the various features and configurations recognized as belonging to the fungus in diseased tissues. In the present work, microhyphae generally stained well with cotton blue and thus could be detected easily; in material obtained from infected trees they generally remained colorless and were more difficult to detect. Reticulations in ray cells had earlier been attributed to the activity of the fungus, but this could not be definitely confirmed. It is now clear that such reticulations can originate from growth of the fungus around starch grains; this fact may have an important bearing in the pathogenesis of the disease.

The present observations also give further evidence that *C. ulmi* may produce wall-degrading enzymes, as shown by the zone of reactions around many of the hyphae.

Further morphological evidence has been obtained that microspore-like bodies can be formed within hyphae and spores as well as exogenously on microhyphae (see Figs. 13, 25, 26). These small spore-like bodies, and endohyphae of various sizes seem to result from a tendency of the protoplasm in hyphae and spores to condense and fragment. Considering that free protoplasts have been obtained from several fungi (14), the phenomenon of "microendospores" (11) does not seem so aberrant. Just how this occurs, however, and how much of the cytoplasm a nucleus needs to reproduce the fungus is a more challenging question.

General Discussion

The technique described in this paper permits one to follow clearly the development of various fungi in wood. The method offers the following advantages: aseptic conditions are assured; development of the fungus can be observed at various intervals after inoculation and under the desired conditions; since only the fungus elements stain (except newly differentiated cells) its characteristics in wood can be easily recognized; artifacts due to sectioning and staining are almost overcome. Because of the ease and rapidity of the operation, it may be used for classification of fungi causing soft rot of wood, and the study of factors favoring wood degradation, or resistance to degradation. Applied to the study of *C. ulmi*, the technique will not only enable a better recognition of the various forms of the fungus in wood but may also prove to be of great value in the search for factors of resistance of elm to the disease. The technique will be useful in screening for fungicides or studying the physiological conditions of both the pathogen and the tree, and undoubtedly, in the investigation of many other problems.

Acknowledgments

This work was conducted by the authors at the Institut für spezielle Botanik, ETH, Zürich, respectively as Graduate Assistant and Academic Guest. Prof. H. Kern and Dr. E. Müller provided help and facilities. The technical assistance of Miss Bertha Aebi and Mr. H. Hofstetter was appreciated. Robert Gagnon, Forest Research Laboratory, Quebec, kindly did most of the photography work.

1. Bailey, I. W., and M. R. Vestal. 1937. The significance of certain wood-destroying fungi in the study of the enzymatic hydrolysis of cellulose. J. Arnold Arboretum Harvard Univ. **18**: 196–205.
2. Courtois, H. 1963. Mikromorphologische Befallsymptome beim Holzabbau durch Moderfäulepilze. Holzforsch. Holzverwert. **15**: 88–101.

3. LIESE, W. 1959. Die Moderfäule, eine neue Krankheit des Holzes. Naturwiss. Rundsch. **12**: 419–425.
4. LIESE, W. 1959. Untersuchungen über das Vorkommen der Moderfäule in Holzschwellen. Holzforsch. Holzverwert. **12**: 61–64.
5. LIESE, W. 1964. Ueber den Abbau verholzter Zellwände durch Moderfäule. Holz Roh Werkst. **22**: 289–295.
6. LIESE, W., und H. PECHMAN. 1959. Untersuchungen über den Einfluss von Moderfaülepilzen auf die Holzfestigkeit. Forstwiss. Centralb. **78**: 271–279.
7. LIESE, W., und R. SCHMID. 1964. Ueber das Wachstum von Bläuepilzen durch verholzte Zellwände. Phytopathol. Z. **51**: 385–393.
8. OUELLETTE, G. B. 1960. Studies on host and pathogen in relation to the infection process in the Dutch elm disease. Ph.D. Thesis, Cornell University, Ithaca, N.Y., U.S.A. p. 123.
9. OUELLETTE, G. B. 1962. Morphological characteristics of *Ceratocystis ulmi* (Buism.) C. Moreau in American elm trees. Can. J. Bot. **40**: 1463–1466.
10. OUELLETTE, G. B. 1962. Studies on the infection process of *Ceratocystis ulmi* (Buism.) C. Moreau in American elm trees. Can. J. Bot. **40**: 1567–1575.
11. OUELLETTE, G. B., and C. GAGNON. 1960. Formation of microendospores in *Ceratocystis ulmi* (Buism.) C. Moreau. Can. J. Bot. **38**: 235–241.
12. RITCHIE, D. 1967. Penetration of wood cells by special extension of Pestalotia hyphae. Mycologia, **59**: 417–422.
13. SAVORY, J. G. 1954. Breakdown of timber by Ascomycetes and Fungi Imperfecti. Ann. Appl. Biol. **41**: 336–347.
14. VILLANUEVA, J. R. 1966. Protoplasts of fungi. *In* The fungi: the fungal organism. Vol. II. *Edited by* G. C. Ainsworth and A. S. Sussman. Academic Press, N.Y. p. 805.
15. VUILLEMIN, P. 1931. Les champignons parasites et les mycoses de l'homme. Lechevalier, Paris. p. 290.
16. ZYCHA, H. 1964. Einwirkung einiger Moderfaüle-Pilze auf Buchenholz. Holz Roh Werkst. **22**: 37–42.